Innate immune reactivity to dental alloys

Rachmawati, D.

2016

document version
Publisher's PDF, also known as Version of record

CHAPTER 1

General introduction

Contents:

1. OPENING REMARKS

The number of metals to which humans are exposed has sharply increased during the 20th century. Metals are ubiquitous elements in the environment. Some metals are natural contaminants of drinking water and food. Additional sources of metals include industrial, agricultural and pharmaceutical products (Thyssen et al. 2007). Metals are undeniably important in our everyday lives from cooking pots and cars, jewelry, coins, window grills and vaccines, to medical and dental devices (Tchounwou et al. 2012; Kettelarij et al. 2014).
In dentistry usually alloys are used which consist of mixtures of two or more metals. Alloying allows for combining different metals in order to get the best properties for particular purposes e.g. inlays, long span bridges, removable partial denture framework, full denture bases, and implants. Pure metals are rarely used in dentistry since these lack sufficient physical and mechanical resistance against masticatory forces. Precious metals such as gold, palladium and platinum have been used for decades in high noble or noble alloys as materials for dental constructions due to their corrosion and tarnish-resistance as well as their relatively good biocompatibility (Anusavice et al. 2013).

Classification of dental alloys has recently been renewed after the introduction of titanium (Ti) and titanium alloys (Table 1). The latter have been located in between high noble and noble alloys because of their excellent biocompatibility which makes these particularly suitable for dental implants and prostheses (ADA 2003). The costs of such alloys are, however, still relatively high as compared to other alloys with similar physical and mechanical properties such as nickel chromium (Ni-Cr) and cobalt chromium (Co-Cr) alloys (Sakaguchi and Powers 2012). The development of these predominantly base metal-based alloys (non-noble or non-precious alloys) has gained weight since the last twenty years, not only because of cost-effectiveness concerns, but also because of the necessity to develop dental alloys with even better physical and mechanical properties (Al-Hiyasat and Darmani 2005)
Base metal alloys contain no or less than 25% noble metal i.e. gold (Au), silver (Ag), platinum (Pt) and palladium (Pd) (ADA 2003). Compositions have now been defined that make alloys not only cheaper, but also corrosion and color resistant, with hardness and elasticity twice higher than most precious alloys. However, the incidence of metal allergies and other side effects from these alloys are reportedly higher than from noble alloys (Anusavice et al. 2013).

In this thesis, the effects of a broad panel of orally applied metals and alloys on the human immune system are described, with a focus on innate responsiveness and early inflammatory events. Of note, these might eventually facilitate the development of allergy and autoimmunity. Knowledge of the relation between oral metal exposure and allergy or autoimmunity serves a public health interest and is valuable for our understanding of mechanisms of action of metal immunotoxicity in general. Reviews and research reports on individual metals and their relations to specific allergies and/or autoimmune diseases (AIDs)

Table 1. Classification of dental alloys based on ADA classifications (ADA 2003)

Classification	Composition	Type of alloy	Usage
High noble alloys	≥ 60% (Au + Pt group*) or ≥ 40% Au	Au-Pt	metal-ceramic restorations
		Au-Pd	crowns, bridges
		Au-Cu-Ag	crowns, bridges
Titanium Alloys	≥ 85% Ti	cpTi (Commercial pure titanium)	Implants
		Ti-6A1-4V (Ti alloys)	metal-ceramic FDPs
Noble Alloys	≥ 25% (Au + Pt group*)	Ag-Au-Cu	crowns, bridges
		Pd-Cu	crowns, bridges
		Ag-Pd	crowns, bridges
Predominantly Base Alloys	< 25% (Au + Pt group*)	Ni-Cr	crowns, bridges, orthod. braces
		Co-Cr	crowns, bridges
		Stainless steel	pediatric crowns, orthod. braces

*Pt group: platinum, palladium, rhodium, iridium, osmium and ruthenium

can be found, but an overview on innate immune responses to clinically relevant transition metals, in particular those neighbouring nickel in the Periodic Table (Chromium (Cr), iron (Fe), cobalt (Co), copper (Cu), zinc (Zn), gold (Au), mercury (Hg), Pd: Figure 1), is lacking. Here we aim to provide such an overview.

2. METALS AND THEIR APPLICATION IN DENTAL ALLOYS

Most elements in the middle part of the Periodic Table (Figure 1) are metals which in dentistry are frequently alloyed together for many purposes, such as fillings, posts/core, crowns, bridges, orthodontic devices. Thus, more than 25 elements in the periodic table of elements are used in dental alloys, but most prominent metals are Ti, Cr, Fe, Co, Ni, Cu, Zn, Pd, Au, Hg (Wataha 2000).

Each metal has its own role, e.g. adding hardness, corrosion resistance etc, when alloyed with other metals to create dental constructions. Whereas nickel has been recognized as an important sensitizer for many decades, the neighbouring elements have now also become notorious for their health effects, e.g. cobalt, palladium and, to a lesser extent, copper (Fage et al. 2014; Yoshihisa et al. 2015). Interestingly, other metals, e.g. Zn, Fe, are known as typical non-sensitizers. The sensitizing level of metals depends on many factors such as their capacity to associate with proteins, i.e. their chemical characteristics and at the other end the available T cell repertoire in the host (Esser et al. 2014). In recent years also the importance of their capacity to trigger early, innate immune responsiveness became clear (Martin 2015). Here, general properties of the selected metal panel are outlined first, after which immunological aspects will be addressed.

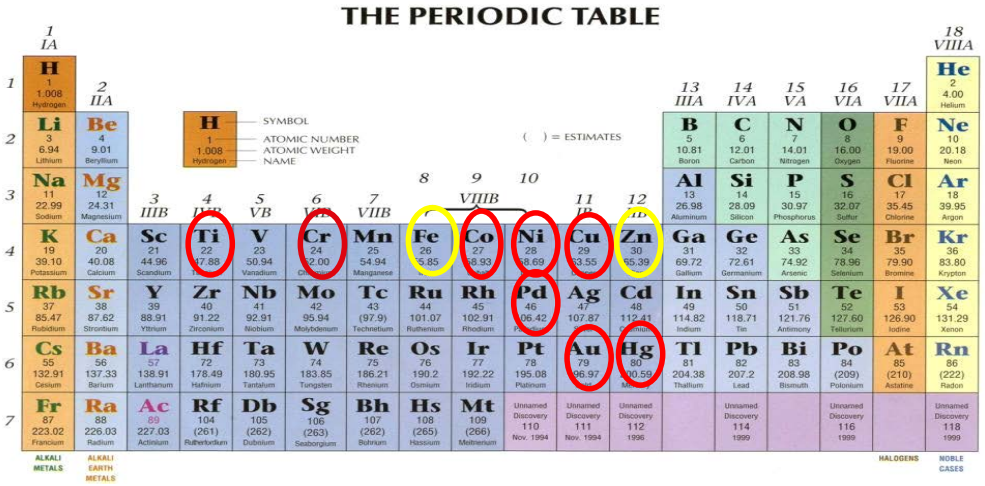

Figure1. Periodic table of elements. Encircled are the metals which we studied in this work. The red circles show the metals which are known as sensitizers whereas the yellow ones show non allergenic metals

Titanium (Ti) is a noble metal. Titanium and titanium alloys, in line with their physical and chemical properties, are clinically suitable for dental implants and prostheses. Due to their biocompatibility, mechanical properties and corrosion resistance Ti alloys are widely accepted as a material of choice for endosseus implant devices, crown and bridge prostheses. However, careful selection of processing methods and laboratory skills are required to guarantee success because titanium is a very hard metal. Ti is generally considered as "biologically inert," although recent research has shown that Ti-based implants undergo biocorrosion and release metal ions and particles into surrounding tissues. Indeed, potential immunotoxicological reactions to Ti receive increasing attention (ADA 2003; Hosoki et al. 2009; Anusavice et al. 2013; Syed et al. 2015).

Chromium (Cr) is widely used in dentistry since it increases the strength of the alloy product, adds durability against corrosion and oxidation, and acts in combination with carbon (about 0.1%) as a hardener. It is usually alloyed together with Ni and/or Co to produce a good quality of alloy. On the other hand, Cr is also known as the most frequent metal sensitizer, after nickel and cobalt (Thyssen and Menne 2010; Yoshihisa and Shimizu 2012). Sensitization is mostly caused by trivalent (3+) metal-ions, which can arise from both metallic and hexavalent (6+) chromium by oxidation and reduction processes, respectively. Chromium is a common commodity in metal industries and its protein-binding capacity is still exploited in the third world leather industries.

Iron (Fe) is an essential micronutrient, vital to metabolically active and proliferating cells. It functions as an oxygen carrier, an electron-acceptor and -donor and as a cofactor for

several enzymes. In the oral cavity Fe is mostly used in gold-platinum based PFM (porcelain fused to metal) alloys. It is used as a strengthener and promotes formation of a porcelain bonding oxide layer. Importantly, its application in stainless steel alloys is rapidly on the rise. Stainless steel alloys are composed of iron, carbon, chromium, nickel, manganese and other metals. The term stainless steel is used when the chromium contents exceeds 11% (usually a range of 12 to 30%). Little is known on possible effects of Fe exposure on allergy and autoimmunity.

Cobalt (Co) is like iron abundantly found in soil, dust, and seawater. In dentistry cobalt has been alloyed with Cr since the twenties, resulting in lower costs and better mechanical and physical properties. This alloy is still commonly used in metal-ceramic crowns and partial denture frameworks. Its immunotoxicological disrepute connects to that of nickel. Contact with cobalt can result in allergic reactions involving cobalt-specific allergic contact dermatitis next to irritant dermatitis. Nevertheless, allergic reactions to Co alloys may not only be caused by cobalt sensitization since these alloys almost always also contain nickel and/or chromium.

Nickel (Ni) is a very important transition element essential for many dental alloys. Nickel-based (non-precious) alloys are used in the dental industry for all types of restorative work (fillings, crowns, bridges, partial dentures) and orthodontic appliances (wires, bands, brackets, etc.). Nowadays nickel is still being used in dentistry for several reasons e.g. corrosion resistance and low cost. Small amounts of nickel improve gold hardness. It is used in dental constructions ranging from a few percent to over 50%. The use of nickel casting alloys for long-term restorations in dentistry has long been controversial since nickel is considered the most frequent metal sensitizer in humans (Rustemeyer and Frosch 2000). In sensitized individuals, a low dose of nickel (about 300 µg or 10 µM) is capable to induce skin inflammatory reactions (Spiewak et al. 2007)

Copper (Cu) is an essential nutrient that in living matter occurs in two oxidation states: cuprous (Cu1+) and cupric (Cu2+) ions. Cu is found in various kinds of food and in drinking water. In dentistry, it is used mostly in crowns and bridges together with gold. Its main role is hardening and strengthening the alloy. In a recent review it was concluded that copper is a relatively weak sensitizer compared to other metals, although in some individuals copper may show severe allergic reactions (Fage et al. 2014). Since copper can cross-react with nickel (Pistoor et al. 1995) it is still unknown to what extent it can act as sensitizer itself.
Zinc (Zn) Similar to Fe and Cu, Zn is an essential micronutrient, with known functions in several parts of the immune system. In dentistry, it is being used in crown and bridge alloys, predominantly as an oxygen scavenger. It excludes oxygen and prevents gas porosity during the casting process. It has also a role in hardening and strengthening the alloy. In porcelain fused to metal (PFM) zinc also lowers the melting range, increases strength and hardness,

and raises the thermal expansion. Zinc plays a central role in the immune system as essential micronutrient for both cellular and humoral immune responses. It is known as a non-sensitizer, although one severe case of systemic contact dermatitis was reported due to zinc-containing dental fillings (Yoshihisa et al. 2015). Such sensitizing capacity, however, still needs to be confirmed.

Palladium (Pd) is also a noble metal and belongs to the platinum group of metals (PGMs) which have similar chemical and physical properties. Exposure to Pd occurs through dental appliances, jewelry, food, inhalation and in some regions through mining pollution. In dentistry Pd has been used widely as a substitute for platinum and gold. It is hard, very strong, white and has a high melting point. It has a very high modulus of elasticity and is also known to provide corrosion resistance. Therefore, Pd is increasingly used for alloying with Au in many dental alloys as they complement each other. However, Pd is also increasingly observed to cause sensitization, up to 10% among the general population in several studies (Hosoki et al. 2009; Faurschou et al. 2011; Muris et al. 2015) Pd is clearly an important sensitizer on its own, but sensitization rates are increased further due to its cross-reactivity with Ni (Pistoor et al. 1995; Muris et al. 2015).

Gold (Au) is a precious, noble metal, known as one of the oldest dental restorative materials and still used for crowns by dentists today. It was assumed to be inert, resisting corrosion. However, it is now known that gold can cause immunotoxicological reactions including sensitization and allergic reactivity (Moller 2002; Nonaka et al. 2003). Contrary to Pd, Au has physical properties as soft, ductile and yellow colored with a low melting point. Thus, the pure metal lacks adequate strength to stand up to the forces generated in the oral cavity. It cannot be bonded to porcelain. Therefore it should first be alloyed with other metals.

Mercury (Hg) has been widely used in dentistry for more than 150 years, due to low cost, ease of application, ideal mechanical properties and durability in the oral cavity. Despite reduced use of mercury in dental applications release of inorganic Hg from dental amalgam is still the most important source of Hg exposure in the western world. Allergic reactions to amalgam fillings are rare but widely confirmed (Schmalz 2002; Aminzadeh and Etminan 2007; Anusavice et al. 2013; Bergdahl et al. 2013; Syed et al. 2015). For several decades possible immunotoxicities of mercury have received much attention, although mechanisms are still not clear whether it is causing allergy, toxicity and/or autoimmunity. Of all Hg exposures, the safety of Hg in amalgam dental restorations has especially received much attention in both scientific literature as well as politics and lay press. Notably, of the total blood Hg, 25% was inorganic, correlating with the number of amalgam fillings and 75% was organic, correlating with fish consumption in a study on Swedish women (Oskarsson et al. 1996).

3. METAL ION RELEASE FROM DENTAL ALLOYS

Dental alloys for dental prostheses have been routinely used in clinical dentistry since the 1800s. As compared to more recently introduced dental materials, e.g. ceramics, composites and polymers, metals provide superior mechanical and physical properties characterized by high strength, ductility, hardness and fracture toughness, needed to withstand chewing stress in the oral cavity (Upadhyay et al. 2006). Next to the mechanical properties, patient safety is a critical factor since dental alloys interact for prolonged times with oral mucosa tissues (periodontal tissues, gingival and alveolar bone) which provide a wet environment facilitating metal ion release (Wataha 2000; Schmalz 2002; Elshahawy et al. 2009). Metal ion release is facilitated by corrosion which is defined as the gradual destruction of materials (usually metals) by chemical reaction with their environment e.g. oxygen. Corrosion degrades the useful properties of materials and structures including strength and appearance, and can be concentrated locally to form pits or crevices. Since corrosion may cause adverse effects, knowledge on the biocompatibility of alloys is of great importance to avoid health problems (Elshahawy et al. 2009; Muris et al. 2014).

Noble metal-based alloys have the longest history of use in dentistry. High noble and noble alloys, in particular gold, platinum and palladium were increasingly used because of their physical and mechanical properties. As a most precious metal, gold was assumed to be relatively inert, resisting corrosion. However, pure gold is weak and therefore needs to be alloyed with other metals. Indeed, mixing gold with copper and other less noble metals resulted in strong, effective filling, crown or bridge alloys. Metal ion release from these alloys remains low and adverse reactions to noble alloys are infrequent. Nevertheless, within the past years gold-allergic symptoms have been reported both in patients with contact dermatitis and in those with oral disease. (Kanerva et al. 2001; Nonaka et al. 2003; Khamaysi et al. 2006; Ditrichova et al. 2007; Davis et al. 2011; Steele et al. 2012; Kim et al. 2015).

Due to the dramatic increase in the price of gold, base metal alloys were introduced to dentistry for dental constructions in the early 1930s. Subsequently, they have largely replaced the noble metal-based alloys for dental prostheses (Nelson et al. 1999; Wataha 2000; Anusavice et al. 2013). When compared with high noble/noble alloys, base metal alloys perform even better as to physical and mechanical properties e.g. high modulus of elasticity which allows thinner metals to be used, and thus minimal tooth destructions during preparation of crown/bridge prostheses (Roach et al. 2000). Alloying conditions and compositions have been explored intensively over the past decades and were optimized for minimal metal leakage and maximal physico-mechanical properties (Lin et al. 2008; Ma and Wu 2011). Nevertheless, doubts remain as to the biocompatibility of the base metal alloys such as Ni-Cr and Co-Cr which tend to have more elevated corrosion rates than the noble alloys. Leakage of metal ions from base metal alloys is also strongly facilitated by handling of the alloys during processing such as casting, heat treatment, and porcelain firing. In

particular recasting of alloys was found to be related to increased metal release. Recasting alloys has been introduced to decrease costs, and involves combining previously cast metal parts e.g. previously melted buttons or sprues removed from casting process, with up to 50% new metal (Al-Hiyasat and Darmani 2005; Imirzalioglu et al. 2012). Micro-structural changes can affect the development of protective surface oxides altering the corrosion behavior of these alloys (Qiu et al. 2011). In brief, base metal alloys are cheap but more difficult to handle and susceptible to corrosion.

Metal release from dental alloys can be tested by classical chemical methods or modern atomic spectroscopic techniques such as Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (Milheiro et al. 2015). *In vitro*, basic measurements are usually made with alloy specimens in water, culture media, or artificial saliva. Virtually all dental mental alloys tested show distinct release rates of metals present in the alloys, generally in the nanogram to microgram range per cm^2 exposed surface area per day (see Table 2). As expected, different alloy types can show markedly different release rates, whereas these rates are not simply correlated with the composition of the alloys (Schmalz et al. 1999; Dimic Ivana D et al. 2014). Moreover, extrinsic factors can increase release rates substantially. In particular low pH values as frequently found in saliva have been shown to accelerate metal release (Dimic Ivana D et al. 2014).

The biologic response to released metal ions does not simply depend on their concentrations but also on the duration of exposure and susceptibility of the host cells (Wataha 2000; Schmalz 2002). Recent studies on tissue-dental alloy interactions therefore involve *in vitro* testing with culture systems utilizing primary cells or cell lines. Here, most frequently used assays are based on cytotoxicity measurements. Of course, *in vivo* studies may provide the most useful data but require appropriate animal models or trial conditions in a clinical setting. Apparently, few animal models have been found of use (mouse, guinea pig, rat) in this regard, whereas important clinical data on metal release rates have become available. Here, measurements have focused on saliva, blood and urine (Sicilia et al. 2008; Elshahawy et al. 2013; Matusiewicz 2014). Also biopsies have been studied from gingival tissues adjacent to metallic restorations showing accumulation of metals in the neighboring tissues (Garhammer et al. 2003). Comparing metal ion release from dental alloys *in vitro* and *in vivo* is difficult since huge variations are found in reported metal ion release data. Clinical variations are most likely due to the fact that numerous factors can affect the release, such as conditions in the oral cavity created by a.o. temperature, pH, saliva, exogenous agents like tobacco and alcohol, ongoing immune reactions, combined presence of different metals as well as the shape and surface treatment of the dental alloys. Also microorganisms or bacterial factors can provide important contributions to corrosion of dental metal alloys. Bacteria inhabit all structures of the oral cavity and easily adhere to alloys (Subramani et al. 2009). On the surfaces of metallic materials they may alter interfacial electrochemical

processes, which can lead to increased corrosion and release of small metal particles or metal salts (Chaturvedi 2013). Next to this plethora of extrinsic factors personal individual factors e.g. dietary habits and live style (Chaturvedi 2009; Mikulewicz et al. 2015) may determine reactions to released metals.

Of note, in none of the reported metal ion release studies the optimal effective innate stimulatory doses found in our studies, i.e. ranging from 125-750 µM for most metals and from 250-750 nM for Hg, were reached. Thus, the amounts of metal ion released from dental appliances in the oral cavity and subsequently absorbed in the gastrointestinal tract are very small. Still, several secondary factors may facilitate the development of systemic complaints (Wataha et al. 2013; Yu et al. 2015). Apparently the wide range of extrinsic factors can act synergistically by enhancing corrosion and metal ion leakage resulting in locally and possibly systemically health-disturbing metal exposure. Whether this leads to substantial local irritancy and/or systemic allergic reactivity may depend on other cofactors, notably concomitant microbial pressure by oral bacteria (de Kivit S et al. 2014). Moreover, while the release of metal ions maybe subliminal it should be realized that it often continues for months or years (Wataha et al. 1999).

4. LOCAL AND SYSTEMIC ADVERSE REACTIVITY TO METAL ALLOYS

Despite the fact that the gastrointestinal tract is known as a tolerogenic 'route d'entrée' for foreign proteins and chemicals, oral exposure to dental alloys has often been associated with local or even systemic adverse reactions. The release of metal ions from dental alloys may be slow but is in fact unstoppable. Depending on their concentration, metal ions can have toxic, inflammatory, allergenic, or even mutagenic effects. Out of these, metal allergies with oral and/or distant lesions are the most well known. The precise pathogenesis of the adverse reactions to metals is, however, often unclear since systemic complaints may be highly unspecific and interpretation of oral lesions is not always easy. In the following paragraphs local and systemic adverse reactions will be described in more detail.

4.1 Local adverse reactions

4.1.1 Oral lichen(oid) lesions

Oral lichen(oid) lesions are common in the general population with a prevalence of 1-2% (Bouquot and Gorlin 1986). They are often observed in association with oral metal exposure, e.g. to amalgam, Au or Pd (Laeijendecker and Van 1994; Koch and Bahmer 1999; Ahlgren et al. 2002; Moller 2002; Laeijendecker et al. 2004). In that case, lesions are mostly unilateral, non-symmetrical and localized on the palate, buccal mucosa or tongue where the lining mucosa comes into contact with restorations. These lesions are referred to as oral lichenoid lesions (OLL). Oral lichen planus (OLP), on the other hand, is putatively caused by an autoimmune reaction involving keratinocytes as target. The lesions are usually symmetrical and also systemic manifestations may occur (Thornhill et al. 2003; Cawson and Odell 2008). Still, the clinical and histopathological aspects of OLP and OLL are indistinguishable, showing

Table 2. Reported metal ion release *in vitro*/*in vivo* from dental alloys

Metal	Type of alloy	Methods & conditions of exposure [a]			Release			Reference [b]
	(name/brand)	device / specimen quantification	fluid volume	duration	µg/cm² /day	Ppb/day (µg/L/day)	nM/day	
Ti	Ti–6Al–4V Zimmer (Sulzer Orthopedics)	dental implant Ø 22 mm x 2mm	MEM [c] 100ml	28 days	<< [d]	<<	<<	(Höhn S and Virtanen S 2015)
	Ti (Ormco)	20 orthodontic brackets 4 buccal molar tubes *(in vitro)*	MEM 30 ml	30 days	n.d. [e]	0.18	3.73	(Ortiz et al. 2011)
Cr	Stainless steel (Ultraminitrim, Dentaurum)	20 orthodontic brackets 4 molar tubes *(in vitro)*	MEM 30ml	30 days	n.d.	0.3	5.76	(Ortiz et al. 2011)
	Ni-Cr (Remanium CS)	cast alloy specimen Ø 5mm x 3mm *(in vitro)*	artificial saliva 11.5ml	60 days	0.0001	0.01	0.192	(Oyar et al. 2014)
	Ni-Cr (Vera Bond II)	cast alloy specimen + electrolysis 40x20x3mm *(in vitro)*	artificial saliva 50ml	2 days	0.085	33.25	639.42	(Galo et al. 2012)
	Co-Cr-Mo (Wironit ®)	cast alloy specimen Ø 8mm x3.2mm *(in vitro)*	artificial saliva pH7.5 artificial saliva pH4 5ml	6 weeks	0.036 0.042	13.09 15.47	251.73 297.5	(Dimic Ivana D et al. 2014)
	Co-Cr (Remanium)	cast alloy specimen + electrolysis 40x20x3mm *(in vitro)*	artificial saliva 50ml	2 days	0.5	18.95	364.5	(Galo et al. 2012)
	Stainless steel (Preform arch wires, Ortho-Organizers)	orthodontic wire 0.017 × 0.025 inch 100 mm length *(in vitro)*	Artificial saliva 100ml	28 days	0.018	0.39	7.57	(Gopikrishnan et al. 2015)
	Stainless steel (American Orthodontics, 3M Unitek)	2 orthodontic wires 20 ortho brackets 2 ligatures *(in vitro)*	artificial saliva flow, 0.5ml/min 19.41 L (flow)	28 days	n.d.	0.01	0.192	(Mikulewicz et al. 2014)
Co	Stainless steel (Ultraminitrim, Dentaurum)	20 orthodontic brackets 4 molar tubes *(in vitro)*	MEM 30ml	30 days	n.d.	0.05	0.84	(Ortiz et al. 2011)
	Co-Cr-Mo (Wironit ®)	cast alloy specimen Ø 8mm x3.2mm *(in vitro)*	artificial saliva pH7.5 artificial saliva pH4 5ml	6 weeks	0.04 0.096	15 34.76	254.23 589.12	(Dimic Ivana D et al. 2014)
	Co-Cr (Remanium)	cast alloy specimen + electrolysis 40x20x3mm *(in vitro)*	artificial saliva 50ml	2 days	0.01	4.65	78.81	(Galo et al. 2012)
	Ni-Cr (Remanium CS)	cast alloy specimen Ø 5mm x 3mm *(in vitro)*	artificial saliva 11.5ml	60 days	0.004	0.35	5.93	(Oyar et al. 2014)
Ni	Stainless steel (Ultraminitrim,Dentaurum)	20 orthodontic brackets 4 molar tubes *(in vitro)*	MEM 30ml	30 days	n.d.	13.9	231.66	(Ortiz et al. 2011)
	Ni-Cr (Remanium CS)	cast alloy specimen Ø 5mm x3.2mm *(in vitro)*	artificial saliva 11.5ml	60 days	0.018	1.38	23	(Oyar et al. 2014)
	Ni-Cr (Vera Bond II)	cast alloy specimen + electrolysis 40x20x3mm *(in vitro)*	artificial saliva 50ml	2 days	0.265	103.5	1.75	(Galo et al. 2012)
	Stainless steel (Preform arch wires, Ortho-Organizers)	orthodontic wire 0.017 × 0.025 inch 100 mm length *(in vitro)*	Artificial saliva 100ml	28 days	0.01	0.21	3.5	(Gopikrishnan et al. 2015)
	Stainless steel (American Orthodontics, 3M Unitek)	2 orthodontic wires 20 ortho brackets 2 ligatures *(in vitro)*	artificial saliva flow, 0.5ml/min 19.41 L	28 days	n.d.	0.035	0.58	(Mikulewicz et al. 2014)
Cu	Stainless steel (American Orthodontics, 3M Unitek)	2 orthodontic wires 20 ortho brackets 2 ligatures *(in vitro)*	artificial saliva flow, 0.5ml/min 19.41 L	28 days	n.d.	0.057	0.904	(Mikulewicz et al. 2014)
	Pd-Cu (Orion Vesta)	cast alloy specimen 2.06cm² *(in vitro)*	lactic acid/NaCl; pH2.3 5ml	7 days	0.37	149.71	2376	(Milheiro et al. 2014)
	Pd-Ag (Orion Argos)	cast alloy specimen 2.06cm² *(in vitro)*	lactic acid/NaCl; pH2.3 5ml	7 days	0.007	2.86	45.39	(Milheiro et al. 2014)
	Amalgam (Tytin (Kerr UK)	discs Ø 10mm x2mm *(in vitro)*	0% H_2O_2 1% H_2O_2 20ml	24 hrs	0.003 0.016	0.38 1.73	6.03 27.46	(Al-Salehi et al. 2007)
	Type IV Au	dental crown *(in vivo)*	Saliva 2ml	3 months	n.d.	0.022	0.349	(Elshahawy et al. 2013)
Zn	Au-Pt based (a.o. Biocclus 4®)	cast alloy specimen plate 10x10x0.5mm *(in vitro)*	H_2O Sprite light®; pH 2.8 20ml	2 hrs	0.366 2.0	40.3 220	610.6 3330	(Johnson et al. 2010)
	Type IV Au	dental crown *(in vivo)*	Saliva 2ml	3 months	n.d.	3.42	51.81	(Elshahawy et al. 2013)
Pd	Pd-Cu (Orion Vesta)	cast alloy specimen 2.06cm² *(in vitro)*	lactic acid/NaCl; pH2.3 5ml	7 days	0.096	39.43	371.98	(Milheiro et al. 2014)
	Pd-Ag (Orion Argos)	cast alloy specimen 2.06cm² *(in vitro)*	lactic acid/NaCl; pH2.3 5ml	7 days	0.047	19.43	183.3	(Milheiro et al. 2014)
	Type IV Au	dental crown *(in vivo)*	Saliva 2ml	3 months	n.d.	0.022	0.207	(Elshahawy et al. 2013)

Table 2. Reported metal ion release *in vitro*/*in vivo* from dental alloys

Au	Au-Pt based (Biocclus 4®)	cast alloy specimen 10x10x0.5mm *(in vitro)*	H_2O Sprite light ®; pH2.8 20ml	2 hrs	0.009 0.068	0.95 7.5	4.84 38.26	(Johnson et al. 2010)
	Type IV Au	dental crown *(in vivo)*	Saliva 2ml	3 months	n.d.	0.022	0.112	(Elshahawy et al. 2013)
Hg	Amalgam (Tytin (Kerr UK)	Discs *(in vitro)* Ø 10mm x2mm	0% H_2O_2 1% H_2O_2	24 hrs	0.0008 0.14	0.11 15	0.54 74.63	(Al-Salehi et al. 2007)
	Amalgam	dental filling *(in vivo)*	Urine	5 years	n.d.	0.44	2.19	(Dunn et al. 2008)

a) Unless otherwise stated, experiments were performed with polished alloys, at 37°, and at a neutral pH
b) References published from 2005 till 2015
c) MEM: Minimal Essential Medium
d) <<.: not detectable
e) n.d.: can not be calculated or not applicable

a variable clinical presentation with white reticular lesions, patches, papules, erosion or ulceration and, when biopsied, dense T cell infiltrates in the oral mucosa underlying the lesions. Although a substantial part of the lichenoid lesions is caused by allergy to dental materials (Cawson and Odell 2008), the pathogenesis of the majority of lichenoid reactions, even if a putative triggering agent has been identified, is not fully understood. It is plausible that also direct immunotoxicity of dentally applied alloys causing local irritant inflammatory reactions plays an important role (Muris et al. 2014).

Most clinicians do not discriminate between OLP and OLL. In case of persistent complaints, replacing restorations adjacent to the lesion is needed to achieve lesion resolution. In addition, patch tests are the gold standard to diagnose the possible involvement of allergy (Thornhill et al. 2003; Issa et al. 2005). The potential premalignant character of metal allergic mucositis is a recent finding (Hougeir et al. 2006; Ismail et al. 2007; van der Meij et al. 2007) and stresses the importance of careful diagnostics and follow up of patients with such lesions (Ditrichova et al. 2007).

4.1.2 Gingivitis/perioral lesions

Typical signs of gingivitis/perioral lesions are redness and swelling that may involve any part of the mouth including the tongue, roof of the mouth, cheeks, and lips (cheilitis). There is occasional formation of blisters and/or ulcers. Affected individuals may complain of a burning sensation and mouth sensitivity to the consumption of or exposure to food, drugs, and other substances (such as metals). This reversible conditions and symptoms resolve once the cause is removed (Dunsche et al. 2003; Ditrichova et al. 2007; Torgerson et al. 2007).

4.1.3 Metal specific allergy

The most common local adverse reaction to metal exposure is metal specific hypersensitivity. This allergy to metals is usually of the delayed type (Type IV in the classification by Gell and

Table 3. Oral problems associated with dental metal alloys

No	Material	Principal elements	Usage	Oral symptoms	Reference
1	Titanium alloys: Ti-Al-V Ti-Al-Nb	± Ti 6%, Al 4-7% , 4% V, 0.25% Fe, 0.3% O	All metal prostheses, Metal-ceramic prostheses, Denture frameworks	Allergy, CFS, Headache, PGCG	(Sicilia et al. 2008; Penarrocha-Diago et al. 2012; Brown et al. 2015)
2	Ni-Cr alloys: Ni-Cr-Mo-Be Ni-Cr-Mo	± Ni 62%, Cr 19.1%, Mo 7.1%, Ga 2%	All metal prostheses, Metal-ceramic prostheses, Denture frameworks	Allergy, AID, Burning mouth, Cheilitis & perioral dermatitis, CSF, Gingivitis, OLP,Orofacial granulomatosis, Stomatitis	(Sterzl et al. 1999; Khamaysi et al. 2006; Torgerson et al. 2007; Stejskal 2014)
3	Co-Cr alloy: Co-Cr-Mo	± Co 63%, Cr 27%, Mo 6%, Other 4%	All metal prostheses, Metal-ceramic prostheses, Denture frameworks,	Allergy , Burning mouth, Cheilitis & perioral dermatitis, Orofacial granulomatosis, OLL/OLP	(Khamaysi et al. 2006; Torgerson et al. 2007)
4	Palladium alloys: Pd-Cu-Ga Pd-Ag-Ga	± Pd 75-61%, Cu 7%, Ag 24-66% In 6.3%, Ga 6%, Ru<1% ·	Metal-ceramic prostheses	Allergy , Burning mouth, Cheilitis & perioral dermatitis, Gingivitis, NPG , OLL/OLP	(Koch and Bahmer 1999; Torgerson et al. 2007; Muris et al. 2015)
5	Stainless steel	± Ni 13-15.5%, Cr 17-19%, Mo 2-4%	Pediatric crown, Orthodontic braces	Allergy, Angular cheilitis, Burning sensation, Numbness, NPGP, Papula peri-oral rash, Stomatitis from mild to severe, erythema	(Rahilly and Price 2003; Genelhu et al. 2005; Kolokitha and Chatzistavrou 2009; Chakravarthi et al. 2012)
6	Amalgam	± Hg (50%), Ag (~22-32%), tin (14%), cu (8%), and other trace metals	Fillings	Allergy, AID, Burning mouth, Cheilitis & perioral dermatitis, CFS, Dry mouth, Fibromyalgia Headache, dizziness,OLP, OLL, Stomatitis	(Koch and Bahmer 1999; Sterzl et al. 1999; Dunsche et al. 2003; Khamaysi et al. 2006; McParland and Warnakulasuriya 2012; Stejskal 2014)
7	Gold alloy Au-Ag-Pd Au-Pd-Cu-Ag	± Au 45-86%, Pd 3-38%, Pt 11.5-12%, Cu 25-10%, Ag 15-14%, Zn 1.6 % In, Rh, Fe each < 1%	All metal prostheses, Metal-ceramic prostheses, Denture frameworks	Allergy, Burning mouth, Cheilitis & perioral dermatitis, Fibromyalgia, Gingivitis, OLP, OLL, Orofacial granulomatosis, Stomatitis	(Koch and Bahmer 1999; Khamaysi et al. 2006; Torgerson et al. 2007; Stejskal 2014; Muris et al. 2015)

CFS : Chronic Fatigue Syndrome
OLP/ OLL : Oral Lichen Planus/ Oral Lichenoid Lessions
NPG : Non-plaque related gingivitis
PGCG : Peripheral giant cell granuloma

Coombs; see paragraph 4.3). It is called 'delayed' because when metals are applied onto the skin of allergic persons, as is done in the diagnostic patch test, the skin becomes red, indurated and inflamed only after 24 till 48 hours, normalizing after 3 or 4 days. Based on such patch test reactivity the dermatologist will diagnose the patient as being allergic for a certain metal or chemical. This type of reactivity is most relevant for the dental clinic, since also exposure via the oral mucosa should be considered as a challenge of the allergic responsiveness. Oral application of Ni containing constructions in a Ni allergic individual may thus cause local inflammation in and around the oral mucosa, but, interestingly, also flaring of former reaction sites even at a distance, e.g. the flare of a former hand eczema (Feilzer et al. 2013). Allergy to orally applied metals such as Ni, Co, and Cr are well known, while other metals like gold and palladium, have recently drawn much attention as important allergens, in particular in patients with oral adverse reactions (Nonaka et al. 2003; Muris et al. 2014). Some other metals (e.g. Zn, Fe) are not known as contact allergens. Table 4 shows the frequency of metal allergies in patients with oral disease. Of the metals tested, Ni, Cr, and Au cause most frequently positive reactions in this population (up to 58.5, 29 and 38.1% resp.). Khamaysi (Khamaysi et al. 2006) examined 134 patients that were patch tested with a dental screen, and evaluated to what extent the clinical manifestations (perioral reactions, burning mouth OLL and other oral complaints) correlated with the allergens for which the patients had positive patch tests. However, no obvious association was found between clinical presentation and particular allergens.

Important for a the diagnostic and therapeutic work up of a patient with adverse skin reactions possibly related to dentally applied alloys is a detailed anamnesis, with focus on allergic reactions and metal exposure, followed by skin testing. Knowledge of the composition of the suspected alloy is essential in this process (Muris and Feilzer 2006). During the years *in vitro* assays with blood samples have been introduced but, although they might add an objective parameter for sensitization in difficult cases, their sensitivity and specificity still is inferior as compared to skin testing (Menne 1981; Skoglund 1994; Rustemeyer et al. 2004).

4.2 Systemic adverse reactions

4.2.1 Autoimmune disease

Genetic and environmental factors play a role in the pathogenesis of AID. Metal exposure could be considered as one of the latter. Still, robust pathogenic studies have mainly been performed in animal models whereas in humans, much controversy exists about the role of metal exposure in the development of autoimmunity.

Several studies suggest a causal relation between exposure to transition metals and autoimmune manifestations in the skin, oral cavity, joints, brain and thyroid gland. Affected individuals may display multiple subjective symptoms associated with several organ systems, the most common being fatigue, fibromyalgia, dizziness and headache. Intra-oral symptoms

Table 4. The frequency of metal allergy in the population with oral disease *a)*

Metal	Frequency	Median Frequency	reference
Ti	- 0% (0/151) [b)]	1.1%	(Torgerson et al. 2007)
	- 2.2% (2/92)		(Davis et al. 2011)
Cr	- 29% (9/31)	13.85%	(Minang et al. 2006)
	- 3.2% (1/183)		(Ditrichova et al. 2007)
	- 0.5% (1/182)		(Torgerson et al. 2007)
	- 10.7% (3/28)		(Steele et al. 2012)
	- 17% (85/500)		(Davis et al. 2011)
	- 22.7% (10/44)		(Kim et al. 2015)
Co	- 38.7% (12/31)	10.2%	(Minang et al. 2006)
	- 4.9% (6/121)		(Khamaysi et al. 2006)
	- 9.7% (3/183)		(Ditrichova et al. 2007)
	- 5.2% (16/307)		(Torgerson et al. 2007)
	- 9.5% (88/931)		(Nonaka et al. 2011)
	- 10.7 (3/28)		(Steele et al. 2012)
	- 14.9% (143/959)		(Davis et al. 2011)
	- 15.9% (7/44)		(Kim et al. 2015)
Ni	- 54.8% (19/31)	19.8%	(Minang et al. 2006)
	- 13.2% (16/121)		(Khamaysi et al. 2006)
	- 12.9% (4/183)		(Ditrichova et al. 2007)
	- 12.5% (40/320)		(Torgerson et al. 2007)
	- 58.5% (148/253)		(Nonaka et al. 2011)
	- 28.6% (8/28)		(Steele et al. 2012)
	- 14.6% (243/1693)		(Kanerva et al. 2001)
	- 25% (11/44)		(Kim et al. 2015)
Cu	- 3.6% (1/28)	3.53%	(Steele et al. 2012)
	- 3.53% (94/2660)		(Wohrl et al. 2001)
	- 0.2% (6/2611)		(Kanerva et al. 2001)
Zn	- 0% (0/903)	0.6%	(Kanerva et al. 2001)
	- 0.6% (1/180)		(Torgerson et al. 2007)
	- 3.5% (34/963)		(Davis et al. 2011)
Pd	- 4.2% (107/2405)	9.7%	(Kanerva et al. 2001)
	- 7.4% (9/121)		(Khamaysi et al. 2006)
	- 12.9% (4/183)		(Ditrichova et al. 2007)
	- 9.7% (19/196)		(Torgerson et al. 2007)
	- 35% (7/25)		(Muris et al. 2015)
	- 25% (7/28)		(Steele et al. 2012)
	- 6.8% (3/44)		(Kim et al. 2015)
Au	- 7.7% (345/4508)	12.8%	(Kanerva et al. 2001)
	- 14% (17/121)		(Khamaysi et al. 2006)
	- 6.4% (2/183)		(Ditrichova et al. 2007)
	- 11.6% (34/293)		(Torgerson et al. 2007)
	- 38.1% (21/55)		(Nonaka et al. 2011)
	- 28.6% (8/28)		(Steele et al. 2012)
	- 28%(306/1092)		(Davis et al. 2011)
	- 4.5% (2/44)		(Kim et al. 2015)
Hg	- 8.9% (139/1568)	9.7%	(Kanerva et al. 2001)
	- 10% (12/121)		(Khamaysi et al. 2006)
	- 19.3% (6/183)		(Ditrichova et al. 2007)
	- 2% (4/198)		(Torgerson et al. 2007)
	- 20.4% (11/54)		(Nonaka et al. 2011)
	- 10.7% (3/28)		(Steele et al. 2012)
	- 9.4% (104/1103)		(Davis et al. 2011)
	- 4.5% (2/44)		(Kim et al. 2015)

a) reports on patch testing of major types of metals from dental alloys; the list may not be exhaustive

b) positive patch tests/total patients tested

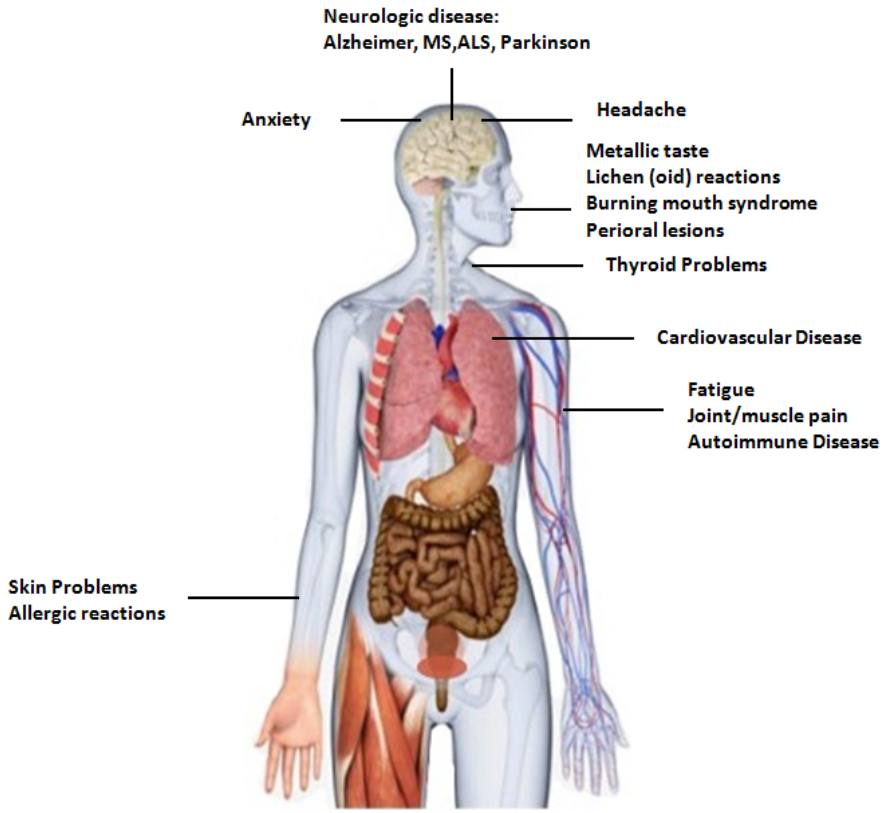

Figure 2. Clinical symptoms which can be related to dental alloys

include burning sensations, taste disturbances and dry mouth (Carocci et al. 2014; Giacoppo et al. 2014; Stejskal 2014).

A direct causative role of metal exposure in the development of AID is, however, difficult to prove. Most studies are epidemiological, relating metal exposure to the frequency of, often subjective, AID manifestations. Hg and Ni exposure were thus considered as potential risk factors for fatigue and autoimmunity (Sterzl et al. 1999). Also Pd has been associated with systemic complaints, such as chronic fatigue and urine Pd levels with thyroid disease (Helm 2002; Torgerson et al. 2007; Muris et al. 2015). A pathogenic role of amalgam in AID is similarly controversial, a meta-analysis on multiple sclerosis (Aminzadeh and Etminan 2007) was not conclusive. Still, some indirect evidence for involvement of metal exposure in autoimmune manifestations is available, since removal of metal constructions may reportedly improve subjective and incidentally objective autoimmune manifestations (Langworth et al. 2002; Lygre et al. 2003; Sjursen et al. 2015).

4.2.2 Neurotoxicity

For many years, neurotoxic effects such as headache, anxiety and disorientation, have been ascribed to metal exposure. Metals may also be involved in the pathogenesis of

neurodegenerative diseases (Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and multiple sclerosis), since Mg, Al, Cd, Co, Cu, Zn and Pb levels were all found to be increased in CSF of these patients as compared to blood plasma, indicating mechanisms of accumulation (Roos et al. 2013). How exactly the entrance and accumulation of the different metal ions into the central nervous system is effectuated still needs to be elucidated (Modgil et al. 2014). Nevertheless, hypothetically, the development of neurodegenerative diseases could be facilitated by augmented metal release from dental alloys in the oral cavity with metals reaching the brain and causing activation of brain microglia cells.

To date, little attention has been devoted to the effects on human brain of the combination of bacteria or bacterial products and metal alloys in the oral cavity. Interaction between bacteria and metal alloys not only facilitates corrosion of the latter and subsequent metal release, but might also result in synergy at the level of inflammation induction, as described in more detail below (see paragraph 4.2).

4.2.3 Chronic Fatigue Syndrome

Chronic Fatigue Syndrome is an idiopathic disease for which, like for most other idiopathic illnesses, such as autism or fibromyalgia, many theories are presented to explain their pathogenesis. Exposure to metals is one of the factors which may be involved. Chronic mercury toxicity is one of the most common factors believed to be a cause of, or contributor to, Chronic Fatigue Syndrome (Sterzl et al. 1999; Stejskal 2014). Besides Hg exposure, Cd and Ni have been associated with this syndrome (Sterzl et al. 1999; Pacini et al. 2012; Stejskal 2014), but conclusive studies are still lacking.

4.2.4 Cardiovascular disease

The risk of cardiovascular disease was studied in Swedish women in relation to mercury exposure from amalgam and fish consumption. Interestingly, increased blood levels of Hg predicted a low risk of death and myocardial infarction (Bergdahl et al. 2013). Blood Cd levels, on the other hand, turned out to correlate with the incidence of heart failure in another Swedish study (Borne et al. 2015). The risk of exposure to other metals for developing cardiovascular disease was recently reviewed by (Hampel et al. 2015). They conclude that long-term exposure to transition metals, due to air pollution or industrial contacts, may result in increased systemic inflammation, eventually facilitating cardiovascular events.

5. IMMUNOLOGICAL ASPECTS OF ORAL METAL EXPOSURE

5.1 Oral immune responses to metals

Via the oral cavity many different substances and microorganisms enter the body. Only a minority of these microorganisms is pathogenic and should be prevented from outgrowth and invasion. This is a delicate job for the immune system, which is on one hand it has to be very sensitive to recognize all pathogens that can inflict harm; on the other hand, when

the immune system is too sensitive, it will see harmless substances like metals as foreign, resulting in allergy or see self antigens as pathogens resulting in autoimmune disease.

Metal alloys in the oral cavity constantly interact with the environment, as discussed in paragraph 2, resulting in a continuous release of metal ions, contacting the mucosal epithelium and the oral mucosa-associated immune system. Generally, oral antigen contact, in contrast to skin contact, will lead to immunological tolerance: thus, nickel containing braces were shown to reduce the risk of developing Ni allergy later in life (van Hoogstraten et al. 1991). However, still adverse immunological effects of oral metal exposure can occur due to local conditions or previous sensitizing skin contacts. These vary from direct immunotoxicity to metal specific allergy and possibly autoimmune disease.

The immune response is classically divided into innate and adaptive immunity. The innate immune system provides a fast, unspecific defence mechanism, but cannot always eliminate infectious organisms. The lymphocytes of the adaptive immune system provide a more specific defence and, in addition, by their specific amplification memory is created so that subsequent exposure to the same pathogens can be handled more efficiently. The two key features of the adaptive immune response are thus specificity and memory. The innate and adaptive immune system work together at all levels of the immune response. As will be outlined below, many metals can evoke both innate and adaptive immune responses.

5.2 Innate immune response to metals

Innate immunity is the body's first line of protection against potential microbial, viral, and environmental attacks, whereas the skin and oral mucosa play lead roles in providing the most powerful barriers that we rely on to stay well. In the oral cavity saliva, containing mucus, enzymes and various anti-bacterial agents, covers the mucosal epithelium and thus contributes largely to this barrier function. Other cellular components of the innate immunity include phagocytes e.g. neutrophils and monocytes/macrophages, dendritic cells, mast cells, basophils, eosinophils and natural killer cells. They all 'sense' pathogens by distinct receptors and contribute to elimination of the pathogen.
The key molecular players in innate immunity are the pattern recognition receptors (PRRs) that recognize molecules broadly shared by pathogens, the so called pattern associated molecular patterns (PAMPs). Activation of PRR signaling pathways triggers the nuclear translocation of various transcription factors, including NF-κB, AP-1, IRFs, and C/EBPβ. This leads to the production of inflammatory mediators to coordinate the elimination of pathogens and infected cells. PRRs include Toll-Like Receptors (TLRs) and C-type Lectin Receptors (CLRs) as well as intracellular Nucleotide-binding and Oligomerization Domain (NOD)-Like Receptors (NLRs). Further, Retinoid acid-Inducible Gene I (RIG-I)-Like Receptors (RLRs) and other, not yet grouped receptors like the cytosolic nucleic acid sensors AIM2

(Absent In Melanoma 2) and DAI (DNA-dependent Activator of INF Regulatory Factors) (Murphy 2012).

Chemicals are extremely diverse in the mechanisms that facilitate and trigger immune responses. The capacity of metals to trigger the innate immune system was first reported by (Schmidt et al. 2010) for Ni. They showed that Ni ions could trigger innate immune responses in humans via TLR4 binding, the receptor for bacterial lipopolysaccharide (LPS). An additional paper (Raghavan et al. 2012) showed that also Co could trigger TLR4. Little is still known about other dental metals and about potential triggering of other PRRs by metals.

The first cells in the oral cavity that come into contact with microorganisms are the keratinocytes. Also keratinocytes (KC) express TLRs, thus providing a first layer of defense in skin and mucosa (Baker et al. 2003; Mempel et al. 2003). Accumulation of metal ions from alloys in oral tissues might thus affect the keratinocytes and induce the release of inflammatory mediators. It is, however, still not known in how far mucosal keratinocytes differ from skin keratinocytes with respect to functional TLR expression. In the skin, KC are known to play a role in all phases of allergic contact dermatitis, from the early initiation phase with the elaboration of inflammatory cytokines, that play a role in LC migration, and T cell trafficking, through the height of the inflammatory phase with direct interactions with epidermotrophic T cells, to the resolution phase of allergic contact dermatitis with the production of anti-inflammatory cytokines and regulatory antigen presentation to effector T cells (Gober and Gaspari 2008; Kosten et al. 2015).

The dendritic cells (DC) reside in between the epithelial cells as a network, ready to pick up potential antigens. They need 'danger signals', such as PAMPS to get mobilized and to become fully active by maturation. While maturing, the DC migrate to the draining lymph nodes (LNs), where they present the peptides derived from pathogens or from metal-protein complexes to specific T cells. One of the most abundantly produced cytokines by DC upon stimulation is IL-8. *In vitro*, IL-8 is secreted by DC in response to allergens and not irritants (Toebak et al. 2006). IL-8 is a chemokine which attracts neutrophils and naive T cells. IL-8 release is widely used as a biomarker for the NF-κB pathway. To induce accurate and efficient pathogen-specific immunity, DC adjust their immune response to the type of pathogen. Importantly, the profile of innate PRR signalling largely determines the quality of the DC and thus the type of the subsequent adaptive immune response (paragraph 5.3).

Microglia are the resident innate cells of the brain, derived from monocytes early in development, and make up approximately 10% of the white matter. In contrast to the short half-life of macrophages, microglia can remain for a lifetime. They clear the brain from debris after neurological injury and are readily activated by pathogens. Like DC they express PRRs like TLRs and also bear MHC-class II (Arroyo et al. 2011) enabling them to function as antigen presenting cells (APC), (Kettenmann et al. 2013). The mRNA of TLR 1-9 has been found to be present in human microglia, and at the protein level, the presence of TLR 2, 3 and 4 has been demonstrated. (Gambuzza et al. 2014; Das et al. 2015). When the microglia

cells are stimulated, they release matrix metalloproteinases, reactive oxygen species and other inflammatory factors (Kauppinen et al. 2008), such as IL-8.
The activation of microglia is advantageous under most circumstances. However, if they are over-activated they can damage host tissue, thereby promoting neural death in both cerebral ischemia and neurodegenerative disorders (Klegeris et al. 2007; Kauppinen et al. 2008; Amor et al. 2014). Hypothetically, the development of neurodegenerative disease such as ALS, could thus be facilitated by metal release from dental material in the oral cavity (Mou et al. 2012; Roos et al. 2013).

5.3 Adaptive immune response to metals

Metal ions, like low molecular weight organic chemicals, have to be protein reactive to become immunogenic and to evoke adaptive immune responses. Since metals are too small to function as haptens themselves, coordinate covalent bonding with cellular and matrix proteins, containing preferably Cysteine (Cys) and Histidine (His) residues, creates epitopes that can be recognized by T cells. Such epitopes will thus always consist partly of self-molecules. To become fully immunogenic the free metal ions and/or metal containing complexes should also provide innate immune danger signals to the PRR of the antigen presenting cells (paragraph 4.2), leading to local production of pro-inflammatory cytokines that induce DC maturation and mobilization to the draining lymph nodes. Here the metal (containing complex) is presented by APC in combination with HLA-class I or II molecules to metal specific T-cells (Fig 3).

Specificity is one of the key-features of adaptive immunity and is mediated by T cells. B cells and antibodies are adapted to recognition of more foreign molecules e.g. containing unusual sugar residues as present in bacteria, and do not seem to play a role in metal specific immune responses. If the TCR of a certain T cell recognizes metal-modified peptides on the surface of APC, the T cell can respond with clonal expansion and cytokine production (Fig. 4; (Rudolph et al. 2006; Stockinger et al. 2006)).
The initiation of a primary T cell response, i.e. triggering of naïve T cells, depends on costimulatory signals given by the APC. These involve both cytokine production and expression of surface adhesion molecules, upregulated during maturation, and enable a close, stimulatory contact between APC and specific T cells. The character of the resulting T cell response (inflammatory or regulatory, mucosa or skin seeking) depends on these costimulatory signals as well as on the local microenvironment in the lymph node where sensitization takes place.

Once naïve T-cells have been triggered and expanded, memory T-cells are generated and released into the circulation. As a consequence the frequency of metal specific memory T cells in the blood will increase: the individual is now ‘sensitized’ or ‘hypersensitive’ and will respond promptly with the production of inflammatory mediators upon all further metal

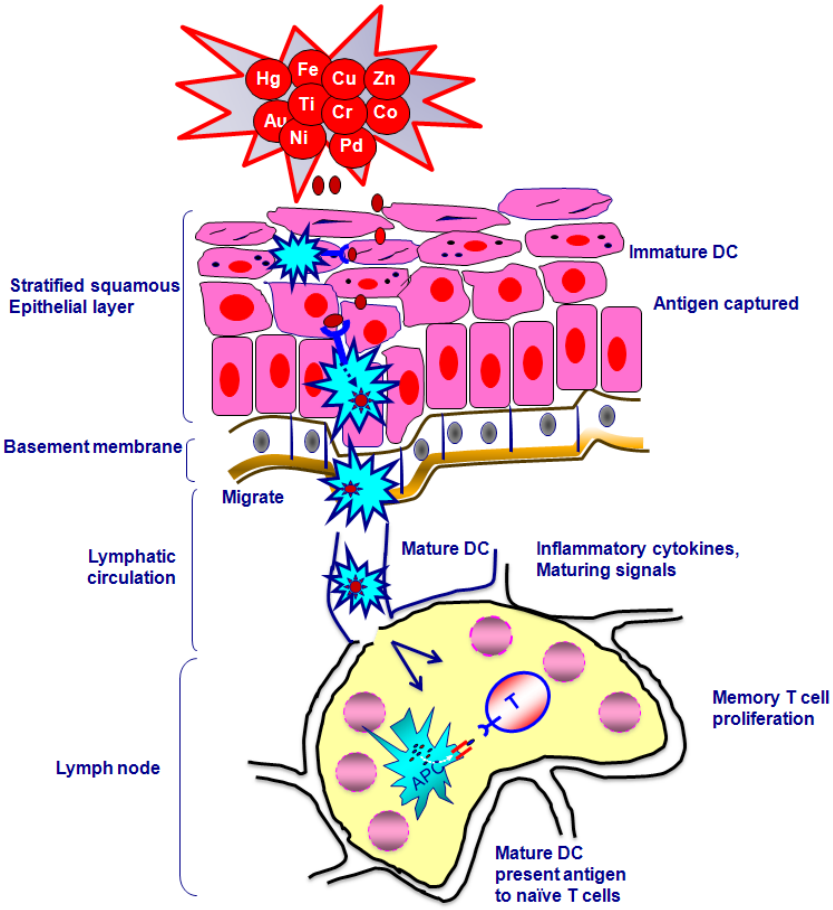

Figure 3. Innate activation by metals resulting in an adaptive immune response.

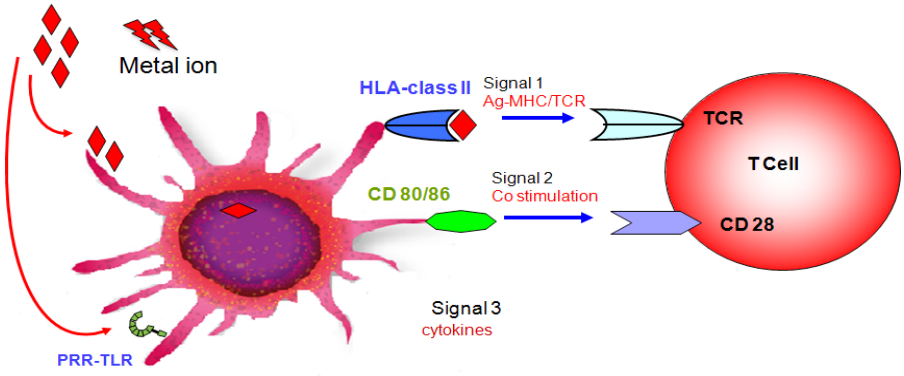

Figure 4. Metal mediated T cell activation: Role of Dendritic cells

contacts. Such allergic reactions can be elicited in the skin but may also take place in the oral mucosa of sensitized patients: upon contact with the relevant metal, T- lymphocytes induce an inflammatory response causing tissue damage, seen as contact dermatitis or mucositis, i.e. intra-oral diffuse, red zones, blisters, or ulceration with pain and burning sensation.
Metal hypersensitivity nowadays is diagnosticated by epicutaneous patch testing with a panel of potentially relevant metal salts in non-toxic concentrations. Skin reactions are read after 48 and 72 hours, since T cell mediated reactions, visible in the skin as erythema, blistering and induration, then reach their maximum. By far, patch testing is the gold standard method for detecting metal sensitization, since it is simple and has minimal adverse reactions. However, the accuracy of this method strongly depends on the experience of the dermatologist who interprets the results. Sometimes irritant reactions are difficult to discriminate from true allergic reactions resulting in 'false positive' read outs. On the other hand false negative patch tests may occur for metals like Ni (46%; (Rustemeyer et al. 2004) and Pd (7.9 %; (Muris et al. 2012) and can be due to still suboptimal formulation of the test salts (Muris et al. 2012) or slow penetration. This relatively lack of accuracy of the skin test together with the fact that each allergen contact may facilitate or boost sensitization stimulated the search for blood-based *in vitro* assays in the last decades (Moed et al. 2005; Muris et al. 2012).

The presence of recirculating metal specific T cells can indeed be demonstrated *in vitro* using peripheral blood lymphocytes. Upon culturing with metal salts, metal-specific lymphocytes will proliferate and produce cytokines, such as IL-5. For diagnosing metal allergy, *in vitro* tests have some advantages as compared to patch tests: they do not interfere with the patient's immune response, they are objective, and can be used in clinical situations where patch testing is contraindicated. However, the quality of the results depends on the number of lymphocytes tested, the metal salt solubility and last but not least the laboratory expertise. In the diagnostics of metal allergy patch tests remain the gold standard, whereas *in vitro* tests are of help in doubtful cases or when skin testing is contraindicated.

5.4 Sensitizing capacity of metals

5.4.1 Allergenicity

The capacity of chemicals to induce an adaptive immune response, referred to as allergenicity, depends on the chemical characteristics as well as on the available T cell repertoire in the host (table 5). The most important chemical characteristics are solubility, capacity to penetrate skin or mucosa, the protein binding capacity and the 'danger signals' they provide to the PRR of dendritic cells, allowing the latter to mature and prime T cells as outlined above. The T cell repertoire in the host is determined in the thymus, where T lymphocytes are educated to optimally recognize pathogen-modified self-molecules. Many different models and test protocols have been developed to predict allergenicity of chemicals, all aiming at a maximal correlation with clinical data from allergic patients. Such

data sets have been published, and for dentally applied metals the frequency of positive patch tests is shown in table 4.

5.4.2 In vivo allergenicity testing

For many years guinea pigs provided the experimental models of choice for testing the skin sensitizing potential of chemicals (Magnusson and Kligman 1969; Maurer et al. 1975). These *in vivo* test systems evaluated whether a chemical can induce an adaptive immune response, as detected by a skin challenge 2-4 weeks later. In 1989, however, the use of mice was introduced as a rapid and cost-effective alternative (Kimber et al. 1989). Soon, the resulting local lymph node assay (LLNA) with focus on the induction phase became the preferred animal model for allergenicity testing. In the LLNA chemicals are applied *in vivo* but the read out is based on lymphocyte proliferation in the draining lymph nodes and measured *in vitro* (Kimber et al. 1989; Dean et al. 2001), Although these models generally predict the sensitizing capacity of chemicals well, reflecting allergic reactivity in men, some common human sensitizers like Ni showed only very weak sensitization in mice. Interestingly, this species dependency could be explained by the findings of Schmidt et al, who showed that the triggering of DC by Ni via TLR4 requires non-conserved histidines at distinct positions, which were not found in the mouse TLR4 (Schmidt et al. 2010). Apparently human cells or tissues would provide optimal test material for *in vitro* allergenicity testing of new metal alloys.

Since 2013 *in vivo* screening methods for cosmetic compounds have been banned, due to ethical and legislative reasons. This has resulted in a renewed interest in quantitative structure-activity relationship (QSAR) models and in *in vitro* tests for allergenicity.

Table 5. Crucial factors for sensitization to metal alloys

	Factor
1.	Exposure to the metal
2.	Metal ion release
3.	Penetration of skin or mucosae by metal (ions)
4.	Protein binding capacity of the metal (ions)
5.	Innate triggering by metal (ions)
6.	Availability of T cells recognizing the metal-peptide complex
7.	Microenvironment in the draining lymph node

5.4.3 QSAR

As outlined in paragraph 4.3 low molecular weight chemicals, including metals must bind to carrier molecules to become antigenic and to be recognized by the immune system. The rate of protein binding is considered a major determinant of allergenic potency (Chipinda

et al. 2011). Whereas small organic allergens usually form covalent bonds with nucleophilic centers on proteins, metals rather bind to proteins through disulfide exchange or coordinate covalent bonds. Based on the assumption that all allergens are protein reactive, screening assays have been developed to predict allergenicity. Such assays use cysteine, lysine, glutathione, or model peptides as carrier molecules and efforts are underway to validate them as alternative *in chemico* methods for screening skin sensitizers (Gerberick et al. 2004; Roberts et al. 2006). The protein binding capacity, together with other chemical characteristics, as a.o. size and solubility (octanol/water partitioning) of the compounds, are used for combined risk analyses of chemicals in so called QSAR models.

5.4.4 Cell based allergenicity testing

As outlined in the previous sections (paragraphs 5.2 and 5.3) a crucial step in the sensitization process is the induction of DC maturation by allergens. This innate stimulatory capacity of chemicals can be tested *in vitro* by exposing immature blood monocyte derived dendritic cells (MoDC) to non-toxic concentrations of chemicals. The resulting *in vitro* maturation of the DC can be evaluated by measuring the release of cytokines by ELISA (IL-8, IL-6 etc) or by monitoring the upregulation of cell surface expression of CD40, CD80 and CD86 by flow cytometry. Nowadays, new technologies such as micro-array or pepchip analysis might also be used to determine allergen-driven DC activation.

Indeed it was shown that allergens, but not irritants could induce maturation of immature MoDC as measured by IL-8 production and upregulation of adhesion molecules (Toebak et al. 2006; Szameit et al. 2008). Also transition metals (Ni> Co> Cu> Pd> Cr) were thus found to stimulate DC (Spiekstra et al. 2005), although the underlying mechanisms were still obscure at that time.

Next to MoDC, various cell lines have been explored as potential DC surrogates including THP-1, a human monocytoid cell line (Miyazawa et al. 2007), to avoid donor-to-donor variability in the assays. Moreover the use of cell lines would allow for robust high throughput screening of potential allergens.

In vivo, DC are always surrounded by keratinocytes and fibroblasts, which are also known to produce cytokines upon allergenic stimuli (Ouwehand et al. 2008; Ouwehand et al. 2010; Haniffa et al. 2013) thereby contributing to the microenvironment in which the DC mature. Therefore, ideally culture systems for allergenicity testing should involve all three cell types (keratinocytes, fibroblasts and DC) (Ouwehand et al. 2011).

6. SCOPE AND OUTLINE OF THESIS

This study addressed the long-standing question why metals differ in their capacity to sensitize. Realizing that innate immune reactivity is key in sensitization, using dentally applied metals and alloys we focused on exploring their capacity to initiate innate imune responsiveness *in vitro*.

In **chapter 2** of this thesis we study a large panel of transition metals, neighbouring nickel in the periodic table of elements for their capacity to activate innate immune cells (monocyte-derived dendritic cells). MoDC activation is monitored by assessment of release of the pro-inflammatory mediator IL-8, a major downstream result from TLR ligation. To identify the mechanisms of innate stimulation by metal salts, different TLR transfected cell lines (HEK293) are used.

In the following **chapter 3** we investigate the *in vitro* responses of blood mononuclear cells and cell lines to metals in order to simplify methods for identification of potential immunotoxicity. The focus of the present investigation is to analyze whether gold and mercury might induce innate immune responses. First dendritic cells generated from culturing peripheral blood monocytes were used.

In **chapter 4** we investigate whether gold, mercury, copper and nickel can induce innate immune responses in keratinocytes (KC). KC play an important role in the local innate immune responses in skin and oral mucosa. In this study we compare the functional TLR expression of oral and skin derived KC.

In **chapter 5** we analyze in how far solid cast alloy specimens as used in dentistry, or the low levels of metal ions released from them can trigger innate cells in the absence or presence of low concentrations of bacterial endotoxin.

In **chapter 6** we analyze the ability of metals to stimulate human brain-microglia cells to induce proinflammatory cytokine production (IL-8), and determine possible mechanisms for such stimulation. In this study we explore whether metals can affect human brain-microglia cells with potential implications for understanding the development of neurodegenerative disorders such as Alzheimer.

In **chapter 7** we study potential relationships between oral metal exposure, metal allergy and autoimmunity (AI) in a well-documented group of 78 individuals. Whether the capacity of distinct transition metals to trigger the innate immune system might facilitate the development of metal-specific allergy or immune responses to simultaneously presented (auto) antigens remains an intriguing question. Here we focus on the putative association between oral exposure to dental alloys and the presence of autoimmunity.

Finally, we will discuss answers to the following specific questions:

1. Is the capacity of metals to stimulate innate immune cells, i.c. dendritic cells, correlated with their sensitizing potential?

2. Can the low metal concentrations released from dental alloys stimulate innate immune cells?

3. Does co-stimulation with microbial molecules, e.g. endotoxin, affect metal-induced innate responses?

4. Are not only dendritic cells but also epithelial cells from skin or oral mucosa showing innate immune activation upon metal exposure?

5. Can metal-exposure induce innate immune reactivity in brain cells, i.c. microglia, and thus contribute to neurotoxicity?

6. Should oral metal exposure be considered a noteworthy risk factor for autoimmune disease?

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